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Role of autophagy in the pathophysiology of nonalcoholic fatty liver disease: A controversial issue

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Abstract

Autophagy is a mechanism involved in cellular homeostasis under basal and stressed conditions delivering cytoplasmic content to the lysosomes for degradation to macronutrients. The potential role of autophagy in disease is increasingly recognised and investigated in the last decade. Nowadays it is commonly accepted that autophagy plays a role in the hepatic lipid metabolism. Hence, dysfunction of autophagy may be an underlying cause of non-alcoholic fatty liver disease. However, controversy of the exact role of autophagy in the lipid metabolism exists: some publications report a lipolytic function of autophagy, whereas others claim a

lipogenic function. This review aims to give an update of the present knowledge on autophagy in the hepatic lipid metabolism, hepatic insulin resistance, steatohepatitis and hepatic fibrogenesis.

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Key words: Non-alcoholic fatty liver disease; Non-alcoholic fatty liver disease; Steatohepatitis; Non-alcoholic steatohepatitis; Autophagy; Lipophagy; Lipid metabolism

Core tip: Autophagy is a mechanism involved in cellular homeostasis. In this review the current knowledge on the role of autophagy in non-alcoholic fatty liver disease (NAFLD) is summarised, with emphasis on the current controversy on the lipolytic *vs* lipogenic function in hepatic lipid metabolism. Furthermore the role of autophagy in the pathophysiology of insulin resistance, hepatocellular injury and fibrogenesis is reviewed to better understand its importance in NAFLD.

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INTRODUCTION

The term autophagy has been introduced by De Duve *et al*^[1] over forty years ago to define a process of vacuolisation for the transport of intracellular material to the lysosomes for degradation. The knowledge and number of autophagy-related publications increased exponentially in the last decade, as the importance of autophagy

in (patho)physiology became recognised. Indeed, autophagy is progressively acknowledged as an important regulator of intracellular homeostasis. Dysfunction of this process has been linked with cardiovascular, respiratory, neurodegenerative and metabolic diseases and with cancer^[2,3].

Non-alcoholic fatty liver disease (NAFLD) is characterised by macrovesicular fat accumulation in more than 5% of the hepatocytes in the absence of known causes of secondary steatosis^[4,5]. This accumulation ranges from scarce to panacinar steatosis and usually starts in Rapaport's zone 3^[6]. It is important to distinguish non-alcoholic fatty liver (NAFL, also known as simple steatosis) from non-alcoholic steatohepatitis (NASH), which is diagnosed when macrovesicular steatosis is accompanied by both hepatocyte ballooning degeneration and lobular inflammation^[5,6]. Simple steatosis has a low risk for the development of advanced disease, while NASH is associated with an increased risk of hepatic and non-hepatic co-morbidities and mortality. NAFLD is epidemiologically linked with obesity and diabetes, and is currently considered as the hepatic manifestation of the metabolic syndrome. Given that the prevalence of these metabolic disorders rises, the prevalence of NAFLD and hence its clinical impact, is rising too^[4,7].

A growing body of evidence indicates that autophagy and lipid metabolism are correlated. Dysfunctional autophagy may therefore contribute to the pathogenesis of NAFLD. However, controversies still exist and the exact role of autophagy in the hepatic lipid metabolism is not entirely elucidated yet. This review aims at summarising current knowledge on autophagy in NAFLD.

AUTOPHAGY

Autophagy is derived from the Greek "auto" and "phagos" and literally means "self-eating". Basal autophagy serves as a housekeeper in the continuous turnover of cellular contents, thereby removing damaged or dysfunctional cellular contents and supplying substrates for energy production. Autophagy can be induced in response to oxidative or metabolic stress^[8,9]. Starvation is commonly used to induce autophagy in research settings. Moreover, liver tissue and hepatocytes are frequently used in research and as such were involved in many major discoveries^[9].

There are three types of autophagy identified in mammalian cells: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy^[2,10,11]. In macroautophagy, cytoplasmic material (*e.g.*, organelles or protein aggregates) is sequestered in a double membrane structure, the autophagosome (Figure 1). This process initiates the formation of a phagophore (also known as isolation membrane), which subsequently lengthens to create an autophagosome. The autophagosome fuses with a lysosome to form an autolysosome where its content will be degraded. When a small portion of cytoplasm is engulfed directly by the lysosome, the term

microautophagy is used. In CMA, proteins that contain a special targeting motif, recognised by heat shock cognate protein 70 (HSC70) and co-chaperones, will be selectively delivered to lysosomes where they are internalised *via* a lysosomal-associated membrane protein 2A (LAMP2A).

Among the three types of autophagy, macroautophagy (hereafter called autophagy) is considered to play the most important role in pathophysiology and is well studied in recent years. Even though autophagy was initially believed to be a non-selective bulky degradation pathway, selective forms such as "mitophagy" (selective autophagy of mitochondria), "peroxiphagy" (peroxisomes), "ribophagy" (ribosomes) or "xenophagy" (invading microbes) are also acknowledged^[2,3,8,12].

The formation of autophagosomes is a dynamic and highly regulated process (Figure 1). It is regulated at the molecular level by autophagy related (*Atg*) genes. These genes were originally identified in yeast, but many orthologues in higher eukaryotes have been found^[2]. A central regulator in autophagy is the mammalian target of rapamycin (mTOR)^[13]. This protein complex inhibits the initiation of autophagosome formation by phosphorylating UNC51-like kinase 1 (ULK1). The class I phosphatidylinositol 3-kinase (PI3K)/AKT pathway stimulates mTOR in response to growth factors, such as insulin. However, under conditions of low energy status the AMP/ATP ratio increases, leading to adenosine 5'-monophosphate-activated protein kinase (AMPK) activation and mTOR inhibition, thereby activating autophagy^[2,13]. The beclin-1/VSP34 (a class III PI3K)-interacting complex mediates nucleation of the phagophore^[2,13]. Two ubiquitin-like conjugated complexes take care of elongation of the formed phagophore into an autophagosome: the ATG5-ATG12-ATG16L1 complex and light chain 3-II (LC3-II). An E1-like protein, ATG7, is a necessary mediator of both conjugation processes, hence an interesting target for the study of autophagy^[14]. LC3 (also known as MAP1LC3) is the major mammalian orthologue of ATG8 and also one of the key regulators in autophagosome formation^[5]. The active conjugated form of LC3, LC3-II, is frequently used as a marker for autophagy in studies^[15]. For further extensive review of autophagy regulation, see references^[11,16].

AUTOPHAGY IN LIPID METABOLISM

Singh *et al*^[17] were the first to convincingly correlate autophagy with the lipid metabolism. They considered it as a novel selective pathway in lipid breakdown and called it 'lipophagy', even though the first clues pointing towards a potential role of autophagy in lipid metabolism were already seen a couple of decades earlier^[18]. In contrast, Shibata *et al*^[19] claimed that autophagy was necessary for the genesis of lipid droplets (LDs) rather than being involved in the breakdown of LDs. Currently, several papers with supporting evidence for both theories have been published. In this section, some common findings will be discussed, followed by reviewing both the oppos-

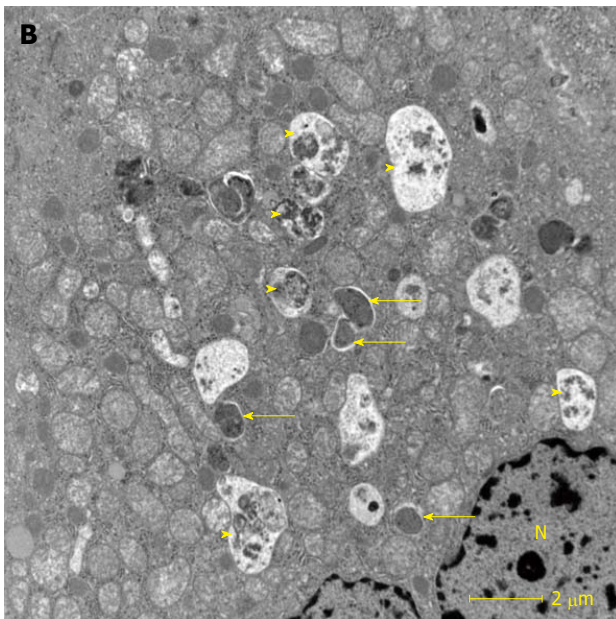
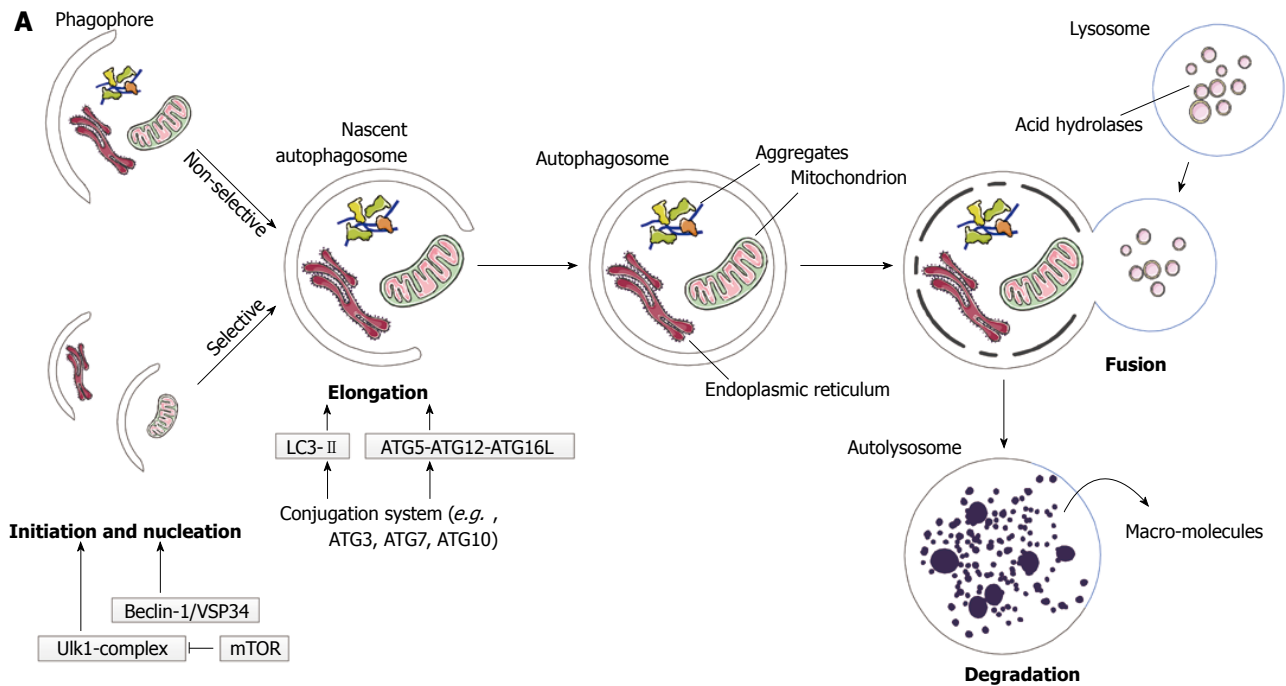


Figure 1 Macroautophagy. A: Schematic overview of macroautophagy. Macroautophagy starts with the formation of a double-layered membrane, the phagophore (isolation membrane). Phagophore formation is regulated by the ULK1 complex (initiation), which is under control of the mammalian target of rapamycin (mTOR) complex, and the beclin-1/VSP34 interacting complex (nucleation). Two major ubiquitine-like conjugated complexes take care of the elongation of the double membrane: light chain 3 (LC3)-II and ATG5-ATG12-ATG16L1. ATG7 is one of the proteins necessary for formation of both elongation complexes. When an autophagosome is formed, it will fuse with a lysosome. The inner membrane of the autophagosome and the sequestered cytoplasm will be degraded and macromolecules can subsequently be (re-)used. Macroautophagy can be non-selective (random uptake of intracellular material) or selective [uptake of specific cargo, e.g., mitochondria, endoplasmic reticulum (ER)]; B: Transmission electron microscopy (TEM)-image of a normal mouse liver fasted overnight. The arrows indicate autophagosomes, the arrowheads indicate autolysosomes. N: Nucleus.

ing theories and the contextual alterations of autophagy in the lipid metabolism.

Common findings in autophagy and lipid metabolism

Despite the contradictory results in the recent literature, some common findings supporting the relationship be-

tween autophagy and the lipid metabolism in the liver deserve to be mentioned.

First, a close relationship between LDs and LC3 has been demonstrated. A notable portion of LC3-positive structures, as demonstrated by immunofluorescence microscopy, co-localised with markers for LDs in liver tis-

sue^[19] and in cell lines^[20-22]. Likewise, immunohistochemistry revealed positive LC3B dots localised on the surface of LDs^[23]. Co-localisation could also be confirmed by LC3 immunogold staining transmission electron microscopy (TEM) and suggests a LD-regulating function of autophagy^[17,19,20]. This co-localisation was not influenced by inhibition of autophagosome-lysosome fusion or knockout of autophagy, suggesting that conjugation to the active form of LC3 (LC3-II) occurs on the surface of LDs and not only on autophagosomes^[17].

Secondly, steatosis is most of the time present in acinar zone 3^[6]. In parallel with this histological finding, immunohistochemical staining for autophagy (staining of LC3) is more localised around the central veins^[23,24]. Zonal distribution of autophagy is also postulated from a theoretical point of view, based on findings in glutamine metabolism^[25]. It is assumed that low rates of autophagy occur in periportal areas and constitutive high levels of autophagy pericentrally in well-nourished conditions. This assumption serves as a potential explanation for the pattern of steatosis in the liver.

Thirdly, LDs have shown to be associated with lysosomes. Immunofluorescence microscopy reveals increased co-localisation of LDs with lysosomal markers such as LAMP1^[17] or lysotracker^[22] in fat-loaden cells. Co-localisation decreases after inhibiting autophagosome formation pharmacologically or by knockdown techniques^[17].

Autophagy as a lipolytic mechanism

Considering autophagy as a lipolytic mechanism is an attractive theory, because it helps explaining the ability of the liver to mobilise free fatty acids (FFA) rapidly, if needed, taking into account that hepatocytes have relatively low concentrations of cytosolic lipases^[26].

Pharmacological inhibition, silencing or knockdown of autophagy (by targeting ATG5) in hepatocytes results in an increased hepatocyte triglyceride (TG) level and accumulation of LDs when cultured in the presence of an exogenous or endogenous lipid stimulus^[17]. It was shown that this increase was due to impaired lipolysis (to fuel β -oxidation) and not to increased TG synthesis. Additionally, the opposite happened after pharmacological induction of autophagy: lipid stores in hepatocytes decreased. The development of hepatocyte-specific autophagy-deficient mice (by targeting *Atg7*) could confirm the *in vitro* results. Indeed, these mice develop hepatomegaly with increased TG and cholesterol content, compared to their wild type counterpartners^[17]. Autophagy-deficient and -competent mice that were starved for 24 h showed an increased presence of TG in their livers (the so-called fasting-induced steatosis). In wild type mice this accumulation was less pronounced. Moreover, TEM demonstrated an increase of lysosomes and lipid-containing autophago(lyso)somes, supporting lipolysis. These findings were even more pronounced with prolonged fasting.

Obese mice (either genetically or dietary) show re-

duced ATG7 protein levels (although the mRNA expression is comparable) as well as decreased levels of autophagy^[27]. Autophagy induction *via* liver-specific overexpression of *Atg7* in *ob/ob* mice improves the metabolic state and reduces the steatosis significantly. These findings further support a lipolytic function of autophagy. Unfortunately, the effects of induced autophagy on lipid metabolism were only reported in *ob/ob* mice and not in high fat diet (HFD)-fed animals.

When blocking autophagy pharmacologically, a decrease in the oxidation of FFA and in the VLDL production was observed, whilst stimulation of autophagy resulted in opposite effects^[28,29]. *In vivo* results also showed a change in the distribution of lysosomal lipases (LAL) towards the autophagosome fraction after starvation, suggesting an increase in the autophagy-mediated lysosomal lipolysis^[28].

Defects in forkhead box class O (FOXO) transcription factors are linked to steatosis and dyslipidaemia^[30]. Mice with a liver-specific triple knockout of FOXO1/3/4 (LTKO) demonstrate steatosis and hypertriglyceridemia^[31]. Reduced autophagy in these mice confirm FOXO1 mediated regulation of the key autophagy genes^[32]. Of note, *Atg14* is regulated by the FOXO transcription factors 1 and 3. Knockdown of hepatic ATG14 increases hepatic and serum TG, whereas overexpression in HFD-fed animals decreases steatosis. In LTKO mice, overexpression of *Atg14* was able to counteract lipid disturbances including steatosis^[31]. In contrast with these experiments, an increase rather than a decrease of FOXO1 levels was described in a small cohort of patients with NASH^[33].

One of the latest contributions in the knowledge of autophagy regulation is the discovery of transcription factor EB (TFEB), which seems to be a master regulator of autophagy^[34]. TFEB is involved in the lipid metabolism as its overexpression inhibits and its suppression induces steatosis, respectively^[35]. The effects on the lipid metabolism were mediated by the stimulation of the peroxisome proliferator-activated receptor γ coactivator 1 α -peroxisome proliferator-activated receptor α (pgc-1 α -PPAR α) pathway as well as by autophagy. Overexpression of TFEB could not counteract steatosis caused by the disruption of autophagy, implying dependency of TFEB function on the autophagy mechanism^[35].

It is generally known that antiretroviral therapy can induce steatosis. In an *in vitro* study, it was shown that the thymidine analogues zidovudine and stavudine inhibit the autophagic flux of hepatocytes in a dose-dependent manner, thereby inducing the accumulation of lipids and mitochondrial dysfunction^[36]. Though not yet used for the treatment of steatosis, glucagon like peptide-1 (GLP-1) analogues were able to reduce endoplasmic reticulum (ER) stress and fat accumulation *in vitro* and *in vivo* by the activation of autophagy^[37]. Carbamazepine and rapamycin induce autophagy and show to be effective in reducing steatosis in models of alcoholic and non-alcoholic fatty liver disease^[38]. Very recently,

caffeine was shown to induce autophagy with increased lipid clearance^[22]. Other possible mechanisms parallel to changes in autophagy that might explain the observed effects are formally not excluded, however, are less likely.

Data in humans are scarce. Studies in human liver tissue are limited partly by experimental restrictions, *e.g.*, the impossibility to use specific pharmacologic interventions or to perform consecutive biopsies. Some currently used markers, such as LC3, do not allow good identification of autophagosomal structures unless the target protein is overexpressed^[23]. Moreover, immunohistochemical markers are a snapshot of a dynamic state and are not able to discern between increased autophagy versus decreased degradation of autophagosomes^[15,23]. Nonetheless, a small immunohistochemical study on post-mortem liver tissue demonstrated decreased LC3 and increased p62 staining with an increased degree of steatosis, suggesting decreased autophagy in more severe steatosis^[24]. Likewise, patients with proven NAFLD demonstrate increased numbers of autophagic vesicles and p62 accumulation on their liver biopsy^[39].

Finally, two clinical observations in patients with NAFLD deserve attention. First, hypothyroidism was found to be more frequent in patients with NAFLD^[40-42]. Thyroid hormone (T₃) is a known regulator of the basal metabolism and acts on different mechanisms. Recently, T₃ was shown to be a powerful inducer of autophagy *in vitro* and *in vivo*, and autophagy accounted for a crucial portion of T₃ stimulated β -oxidation^[21]. As a result, autophagy may provide the explanation for this association.

Secondly, patients with NAFLD have an increased prevalence of hypovitaminosis D^[43]. Vitamin D prevents the development of steatosis, whereas knockout of its receptor (VDR) promotes steatosis^[44]. Others have shown that vitamin D acts as a potent inducer of autophagy^[13]. Indirectly, these two facts together are in line with lipophagy, but hitherto the hypothesis that these effects are directly mediated *via* autophagy has not been investigated.

Autophagy as a lipogenic mechanism

In fasting conditions, the body's energy supply is maintained by adaptive mechanisms. As insulin levels decrease, lipolysis in the adipose tissue (AT) is no longer inhibited and FFA are released in the serum. These FFA are captured by the liver and either used for formation of ketone bodies, or temporarily stored as TG in LDs^[19,45]. In rodents this mechanism may cause substantial accumulation of TG in the liver, known as fasting-induced steatosis. C57Bl/6 mice showed to be very prone to develop steatosis in fasting conditions^[45]. Also in humans, the liver fat content increases on imaging (with ¹H-magnetic resonance spectroscopy) after 36h fasting^[46].

Mice with a hepatocyte-specific autophagy deficiency do not show fasting-induced steatosis as compared to wild type animals. The remaining LDs are much smaller

in number and size and the total TG content in the liver is lower. This lack of fasting-induced steatosis was first seen in very young mice (twenty-two days old), but was confirmed in eight to twelve week old mice^[19,23,47]. Autophagy is therefore implicated in LD formation and growth. The co-localisation of LC3 (necessary for autophagosome formation) with LDs in starved wild type mice further supports these findings^[19].

Subsequent *in vitro* research confirmed these findings in different cell lines (including hepatocytes)^[20]. Cells subjected to knockdown of LC3 form less LDs and have less TG content compared to their controls. Neither FFA uptake, nor TG synthesis or TG breakdown are influenced in LC3 knockdown cells, suggesting an impaired ability to preserve synthesised TG within these cells^[20].

Very recently, an improved metabolic profile was observed in both hepatocyte- or skeletal muscle-specific autophagy-deficient mice^[48]. Adult mice with a deficiency in hepatic autophagy^[47,48] and fed a control diet show reduced fasting-induced steatosis. Moreover, lipid accumulation did not develop^[48] or increase^[47] after feeding a HFD. Gene expression of proteins involved in fatty acid and TG synthesis was lower compared to control littermates. On the other hand, gene expression of proteins involved in β -oxidation and TG secretion was also reduced^[47,48]. It is not clear whether these findings are epiphenomena or directly involved in preventing steatosis. The "mitokine" fibroblast growth factor 21 (FGF21), induced by mitochondrial stress, was held responsible as a central mediator of the metabolic alterations^[48].

Decreased autophagy has been reported in dietary and genetic models of obesity, whereas overexpression of *Atg7* had beneficial metabolic effects^[27] as discussed above. Nevertheless, suppressing *ATG7* expression in lean mice increases hepatic glycogen content, but fails to alter lipid accumulation in the liver (as well as TG or FFA in serum)^[27]. This study is therefore non-conclusive about a lipolytic or lipogenic function of autophagy.

Contextual variability of autophagy in lipid metabolism

Besides the duality in autophagy as a lipolytic or lipogenic process, there are also differences described depending on its context. In most cases these differences were found in experiments supporting autophagy as a lipolytic mechanism.

Based on *in vitro* experiments, basal autophagy is supposed to be a more important pathway for lipid metabolism than induced autophagy^[17]. Hepatocytes in culture did not demonstrate signs of induced autophagy in response to lipid stimuli. Moreover, in contrast with endogenous lipid load, these hepatocytes were unable to adjust the autophagic flow to a sudden increase in the external lipid load. In line with this *in vitro* finding, the external lipid load by prolonged HFD decreases the efficiency of autophagy^[17,27,29,37]. Intriguingly, a biphasic time course is observed with an increase in the autophagic flux and mRNA of autophagic markers after two weeks

HFD and a decrease afterwards (ten weeks HFD)^[29]. Recent data (only presented as an abstract) suggest that autophagy decreases on short term HFD (three days) and normalises after long term HFD (ten weeks)^[49]. Instead of a decrease, eight weeks of a diet high in fat load generates an increase in autophagy^[50]. Paradoxically, a further increase of autophagy by a compound found in garlic decreases the lipid content, possibly by autophagy-independent mechanisms that have to be further elucidated. All together, it seems that alterations of autophagy are dynamic in states of overnutrition.

The term lipotoxicity covers all detrimental effects of fatty acids on the cellular integrity^[51,52]. Hence, it is not surprising that lipids as such can influence autophagy. Short chain fatty acids are able to induce autophagy *in vitro*^[53]. Unsaturated fatty acids (*e.g.*, oleic acid) stimulate autophagy and protect against apoptosis, whilst saturated fatty acids (*e.g.*, palmitic acid) inhibit autophagy and promote apoptosis^[37,54,55].

Not only fatty acids, but also the lipid composition of membranes or vesicular compartments can influence autophagic behaviour. A long exposure to high lipid concentrations alters the membrane composition and diminishes the fusion capacity of autophagosomes and lysosomes^[56]. This may explain the altered autophagy after prolonged fatty diets. Attenuation of CMA was also observed after lipid challenge^[10]. However, some authors did not observe an attenuated fusion capacity in *ob/ob* mice. Instead they report a decrease in clearance of autophagosomes due to a disturbed acidification of lysosomal compartments^[57] and/or down-regulated cathepsin expression^[39,57].

Variation of autophagic behaviour has also been reported with respect to tissue type. For example, autophagy is indispensable for adipogenesis and transdifferentiation of white AT^[58,59], contradicting a potential lipolytic function in AT. These findings are in contrast with findings of autophagy in the liver, which mainly claim a lipolytic function of autophagy (as discussed above). In addition, an increased level of autophagy in AT of patients with metabolic syndrome or type 2 diabetes mellitus is observed^[60-62].

Discrepancies and hypotheses

A clear-cut explanation for the aforementioned discrepancies in autophagy and lipid metabolism is currently lacking. Several hypotheses, however, have been put forward.

Because autophagy declines with age^[63], some concerns were raised about the age of the laboratory animals^[64]. Sometimes, juvenile rather than adult mice were used in experiments^[19], which may be less dependent on autophagy. However, additional experiments with younger and older mice provide similar results^[19,23,47,48], suggesting that the observed differences cannot be explained solely by age.

Secondly, small variances in the mouse strains might be responsible for different outcomes in *in vivo* experi-

ments^[48]. This explanation, however, is even so not likely to offer a solution. Most of the experiments were performed on a C57Bl/6 background, which is an inbred strain. Moreover, tracing back the cited resources of the hepatocyte-specific *Atg7* knockout mice that were used, leads to the same origin of the mice: *Atg7* flox mice (used by^[17,23,65-67]) were created by Komatsu *et al.*^[14] and albumin-Cre mice (used by^[17,23,48,66]) were created by the group of Magnuson^[68]. Furthermore, *in vitro* experiments on hepatocyte cell lines also showed conflicting results^[17,20,36,37].

Thirdly, many different methods can be used to examine lipid accumulation and autophagy. Liver steatosis can be induced by fasting, by genetic and/or by dietary alterations. Different genetic modifications or pharmacological interferences can also alter autophagy. Whereas papers claiming a lipolytic role use a wide range of methods (*e.g.*, *in vitro* methods, pharmacological interference, genetic modifications) (see 3.2), articles claiming the opposite mainly use *in vivo* knockout models and fasting-induced steatosis (see 3.3). However, one of the caveats in knockout models is the potential of influencing developmental stages, *e.g.*, transdifferentiation of white adipocytes requires autophagy^[58,59]. This implies that the observed differences in autophagic lipid handling might be due to the method of inducing fat accumulation or an altered maturation of hepatocytes.

Fourthly, distinction is made between basal and induced autophagy. Basal autophagy is supposed to be the most important type of autophagy in the pathogenesis of NAFLD^[17,69]. However, most studies are performed after total blockage of autophagy, making it difficult to discern between the two. A *Bcl-2* knock-in model is able to selectively block stimulus-induced (*i.e.*, by exercise or starvation) autophagy^[70]. These mice have an impaired glucose-uptake during exercise and an impairment of the exercise-induced protection against glucose-intolerance and increased serum lipid levels caused by HFD. The liver and pancreas morphology did not alter after HFD, supporting the importance of basal autophagy in the lipid metabolism. Further elucidating the role of basal *vs* induced autophagy, including the relationship towards the lipid source, may provide a possible explanation for the divergent findings in the lipid metabolism.

Moreover, when studying autophagy, it is sometimes difficult to distinguish whether the observed effects are a secondary/adaptive process or directly caused by autophagy^[59,71]. Furthermore, autophagy not only influences lipid metabolism, other organelles and cellular systems are also influenced by dysfunctional autophagy and may partly explain observed differences in the liver metabolism (*e.g.*, ER stress and dysfunctional mitochondria^[44,72,73]). Disturbance of very low density lipoprotein (VLDL) production might also be implicated, as autophagy can degrade apoB, a necessary protein for the VLDL formation^[71]. There even may be non-autophagic (and autophagy-independent) functions of ATG-proteins^[26,27,74] in the lipid metabolism.

Inversely, the lipid metabolism is not solely depen-

dent on autophagy alone. Cytosolic lipases still account for a substantial part of the lipolysis^[51]. For example, after total blockage of lipolysis by diethylumbelliferyl phosphate (DEUP), the cellular TG content increases more than after blocking autophagy alone^[17]. Additionally, if LD-formation is autophagy-dependent, small LD-like bodies are observed on TEM in autophagy-deficient cells, suggesting that the lumenally-sorted LD production (*i.e.*, LDs formed out of the ER) is not affected^[19].

Finally, while the resolution limit of microscopical techniques does not allow to visualise the smallest LDs in living cells^[75], it is possible that the observed effects of autophagy only reflect LD modulation after the formation of LDs. Autophagy might be a dynamically active process which controls LD size and the amount of lipotoxic FFA in the cytoplasm. The behaviour of autophagy will be context-dependent and drives the main outcome of autophagy in the lipid metabolism^[51]. In this view, lipolysis and lipogenesis are no longer mutually exclusive and in fact co-exist.

AUTOPHAGY AND INSULIN RESISTANCE

The liver plays a central role in the glucose metabolism and an impaired insulin signalling is an important feature of NAFLD^[4]. Autophagy substantially contributes to maintain the glucose homeostasis and is strictly regulated by insulin^[76]. Similarly to the lipid metabolism, the exact interactions between the action of insulin and autophagy are not entirely clarified yet. An overview of the current knowledge is given in Figure 2.

Insulin stimulates mTOR *via* the class I PI3K /AKT pathway, and thus inhibits autophagy^[2,16]. In case of a normal insulin sensitivity of the liver, insulin-dependent stimulation of this pathway is a possible mechanism of reduced hepatocellular autophagy in hyperinsulinemic states. Furthermore, an alternative pathway was found, which could explain the reduced autophagy in case of insulin resistance (IR)^[32]. In HFD-induced IR, a down-regulation of autophagy was noticed due to a reduced FOXO1-mediated expression of key autophagy genes. The authors suggested that IR was a consequence of the reduced clearance of dysfunctional/aged mitochondria *via* mitophagy, as oxidative stress and altered mitochondrial integrity (and mass) are related to IR^[32]. The impact of other dysfunctional organelles on IR was not investigated, but could also contribute to these findings.

Reduced autophagy was also linked to IR in a study with HFD and *ob/ob* mice^[27]. In contrast to the aforementioned study, IR was not the cause but the result of decreased autophagy. Knockdown of autophagy in lean mice induced severe IR, while overexpression of *Atg7* in obese mice improved the insulin sensitivity and glucose tolerance, decreased the hepatic glucose production and decreased steatosis. An increased level of the calcium-dependent protease calpain 2, which can cleave several autophagy-related proteins, was observed and was held responsible for the decreased protein level of ATG7.

The subsequent increase of cellular stress, with emphasis on ER stress, might be the mechanism behind IR^[27].

Hepatic IR appears to be the result of ER stress-mediated processes^[72], therefore ER stress secondary to decreased autophagy might be a plausible underlying mechanism for IR. Intracellular saturated fatty acids can also contribute to IR by an increase in ER stress, but also *via* an ER-stress independent mechanism^[72]. On the other hand, ER stress stimulates autophagy^[16,77], thus autophagy potentially acts as an escape mechanism to prevent cell injury and IR in particular.

Recently, Kim *et al.*^[48] described a novel endocrine and metabolic function of autophagy. Defective mitophagy causes cellular stress, inducing a stress response regulated by activating transcription factor 4 (ATF4), which promotes the expression of FGF21. FGF21 has several beneficial metabolic effects in lean and HFD-fed animals including an improved glucose tolerance and insulin sensitivity^[48,78]. In human subjects, however, a positive correlation was observed between plasma FGF21 levels, IR^[79] and steatosis^[80]. This apparent paradox might be explained by the resistance to FGF21 (as with increasing levels of FGF21 less IR is actually expected, based on experimental data) or can either be explained by an adaptive increase of FGF21 after establishment of IR. In this study, mitochondrial dysfunction is no longer seen as detrimental, but rather as beneficial by reducing the fasting-induced steatosis and improving the glucose metabolism.

Finally, inhibiting and stimulating effects of protein kinase C (PKC), an important effector enzyme in several signal transduction cascades, on autophagy have been described^[81,82]. Several isoforms of PKC are known and many of them can be activated by diacylglycerol (DG). Insulin resistance is also linked to PKC^[83]. As DG is an intermediate as well as a product of lipolysis, these findings may indicate an additional crosslink between IR and autophagy. Not all DG is able to activate PKC due to differences in stereoisomers. DG produced by lipolysis does not show bioactivity, and therefore potential cross-talk is less likely^[51,83]. On the other hand, active stereoisomers of DG (*i.e.*, 1,2-diacyl-glycerol) can be generated in lipid synthesis and interference with insulin signalling can still occur.

AUTOPHAGY AND HEPATOCELLULAR INJURY

In some patients, steatosis leads to cellular injury and inflammation (NASH) with a subsequent risk for progression to cirrhosis and eventually for hepatocellular carcinoma (HCC) in a subset of patients^[4]. Because damaged organelles are removed by autophagy, dysfunction of autophagy likely will result in cellular injury. In line with this hypothesis, stimulation of autophagy could indeed reduce liver injury in animal models of ethanol-induced steatohepatitis^[38,84]. Carbamazepine-induced autophagy also demonstrated a tendency to reduce cell injury in a

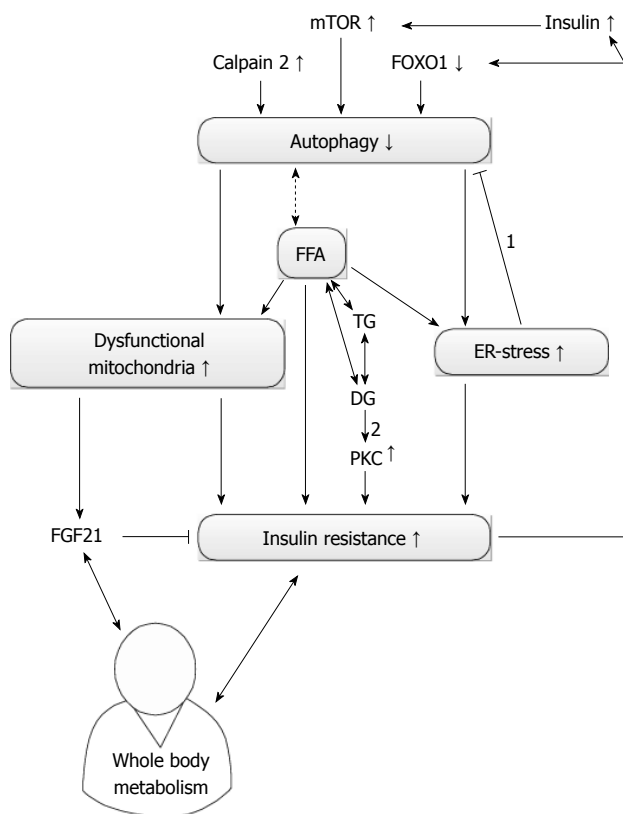


Figure 2 Current knowledge of autophagy and insulin resistance. Autophagy and insulin resistance (IR) seem to influence each other reciprocally. On one hand, an increased level of calpain 2 reduces autophagy and increases IR by increasing endoplasmic reticulum (ER) stress. The amount of dysfunctional mitochondria will also increase and contribute to IR. On the other hand, secondary hyperinsulinism due to IR can decrease autophagy if the insulin sensitivity remains present. Furthermore, autophagy can also be suppressed via an IR-mediated reduction in forkhead box class O 1 (FOXO1). Free fatty acids (FFA) can induce IR directly or by increasing ER stress. Correct stereoisomers of diacylglycerol (DG) can induce protein kinase c (PKC) dependent IR. Controversy exists on how autophagy influences the level of FFA and if subsequent correct stereoisomers of DG can be formed. Dysfunctional mitochondria can increase the level of fibroblast growth factor 21 (FGF21), which is able to reduce IR. FGF21 and IR interact with whole body metabolism. Arrows indicate a consequence of a certain alteration, bar headed arrows denote an inhibition. Double-headed arrows present a reciprocal influence. The dashed and double-headed arrow denotes the uncertain relation between FFA and autophagy. 1: ER stress actually increases autophagy; 2: Only right stereoisomers induce PKC. mTOR: Mammalian target of rapamycin; TG: Triglycerides.

mouse model of NAFLD^[38].

A potential role exists for mitophagy since mitochondrial dysfunction leads to the production of reactive oxygen species (ROS), causes oxidative stress and is involved in the pathogenesis of NASH^[73]. Many of the aforementioned studies do report damaged mitochondria when autophagy is defective^[14,23,36,48,54,66]. In one study defective autophagy was accompanied by an increased production of ROS^[36]. Knockdown of autophagy makes hepatocytes more susceptible to cell death caused by menadione-induced oxidative stress^[69]. This type of cell death is caspase-dependent and activated *via* the mitochondrial death pathway due to c-JUN N-terminal kinase (JNK)/c-JUN overactivation. CMA is upregulated as a compensatory mechanism, but fails to overcome the

induced oxidative stress. Furthermore, CMA as such also provides protection to menadione-induced cell death, but through a different mechanism^[69].

Comparable to menadione, TNF-induced hepatic injury also causes increased cell death, JNK/c-JUN overactivation and activation of the mitochondrial death pathway in hepatocyte-specific autophagy-deficient mice. However, primary mitochondrial dysfunction followed by oxidative stress or impaired energy homeostasis is not responsible for cell injury in this model^[47,65].

The protein p62/SQSTM1 selectively guides proteinaceous aggregates to autophagosomes and accumulates in autophagy-deficient cells. Presence of this protein contributes significantly to hepatocellular injury caused by autophagy deficiency, as double knockouts of autophagy (*Atg7*) and p62 (DKO) diminish hepatocyte injury compared to autophagy knockout (*Atg7*) only^[67]. Nevertheless, p62 is believed to be a beneficial adaptive response to promote formation of relatively harmless aggregates. Toxic intermediates are formed during the aggregate formation and are considered responsible for cell injury. Furthermore, p62 also aids nuclear translocation of the beneficial transcription factor NF-E2 related factor 2 (NRF2), which induces transcription of various detoxifying enzymes^[67]. Of note, despite the alleviation of cellular injury by DKO, a complete abolishment of the cellular injury comparable to control levels cannot be achieved as turnover of disturbed organelles is still not corrected.

Autophagy is linked to the inflammatory cytokines in adipocytes. When p62 is knocked out in adipocytes, an increased invasion of macrophages and production of pro-inflammatory cytokines in AT is observed^[85]. Moreover, knockout of p62 in AT also causes obesity and glucose intolerance, whereas knockout of p62 in hepatocytes does not. Whether these inflammatory changes also occurred in liver tissue was not investigated. A direct inhibition of autophagy in human or mice adipocytes increases the production of pro-inflammatory cytokines^[62] as was described in other tissues as well^[86].

The role of autophagy in tumourigenesis is dual and depends on the stage of tumour development. Autophagy acts as a tumour suppressor in normal tissue and prevents the development of malignant neoplasia. Once a tumour is developed, autophagy drives survival of tumour cells by supplying nutrients^[2]. An extensive discussion of the role(s) of autophagy in liver tumour development can be found in ref.^[9].

AUTOPHAGY IN LIVER FIBROSIS

The knowledge on autophagy in fibrogenesis is scarce, but available evidence suggests an elementary role in different fibrogenic cells^[87]. Autophagy seems to provide nutrients to fuel the processes involved in the activation of these cells.

Hepatic stellate cells (HSCs) are considered major fibrogenic cells in the liver. A central observation in the transdifferentiation from a quiescent state to active myo-

fibroblasts is the depletion of lipid stores, in HSC typically mainly composed of vitamin A^[88].

Autophagy in HSC activation was first evidenced in 2011^[89]. In this study, an increased autophagic flux was observed during HSC activation. Pharmacological inhibition of autophagy inhibited the activation of HSCs. Autophagy also interfered with the LD metabolism after stimulation by platelet derived growth factor BB (PDGF-BB), a known mitogen of HSCs, as shown by co-localisation of LC3B with LDs. Interestingly, autophagy seems to affect only larger LDs and co-localisation became absent once HSCs were activated.

The importance of autophagy in HSC activation was confirmed in another study using pharmacological and genetic tools to inhibit autophagy *in vivo* and *in vitro*^[90]. The results showed an energy-supplying role (*via* delivery of FFA out of LDs for β -oxidation) of autophagy needed for the transdifferentiation of HSCs. Administration of oleic acid in autophagy-deficient stellate cells could partly restore the activation, but did not augment fibrogenesis in autophagy-competent cells.

In line with the aforementioned results, one may expect an increase in fibrosis when inducing autophagy. Paradoxically, reduced hepatic fibrosis is observed after administration of rapamycin, a known potent inducer of autophagy. The involvement of autophagy was, however, not specifically tested in these studies^[91,93]. Rapamycin is known to have a complex mode of action. Hence, studies with other autophagy-inducers may clarify whether the rapamycin-induced reduction in hepatic fibrosis is due to autophagy-related mechanisms.

FUTURE PERSPECTIVES

Further research is clearly needed to elucidate the exact role of autophagy in NAFLD. Fortunately, the increasing research interest in NAFLD and the growing awareness of autophagy as a pathophysiological mechanism will most likely result in new discoveries in the next decade.

The major issue to be resolved is the apparent paradoxical behaviour of autophagy in the lipid metabolism as discussed in this review. At this moment, evidence towards a pro-lipolytic function in the liver is more solid and outnumbers the evidence against this theory. However, the evidence for a lipogenic role cannot be ignored. It has to be addressed whether the two roles might co-exist or whether one role dominates in the hepatocyte, and how their balance and/or function is regulated exactly.

Contextual differences were noted, but not fully understood. Detailed knowledge of basal and induced autophagy may resolve a part of this puzzle. It would be very interesting if future research can more specifically and separately investigate the role of basal and induced autophagy in liver metabolism. Additionally, the relationship with other cellular observations such as dysfunctional mitochondria should be clarified.

Most studies perform experiments on one specific cell type (*i.e.*, hepatocytes), although the liver is com-

posed of many different cell types (*e.g.*, endothelial cells, HSCs and Kupffer cells^[94]). More extensive research on whole liver tissue may be useful as several cell types are also involved in the development of steatohepatitis^[4,88] or may influence each other. Moreover, potential zonal differences of autophagy in liver tissue should be investigated and correlated with the already known different functions of the acinar liver zones^[25].

The liver is not a “cavalier seul” in the metabolism, but one of the central players of the whole body metabolism. The role of autophagy is site-specific. For example, in AT stronger evidence exists for a lipogenic action of autophagy^[58,59] contrary to the results in hepatocytes. In order to understand the complex role of autophagy, other tissues and other metabolic pathways, including the glucose and protein metabolism, must be incorporated in the research projects.

Ultimately, if knowledge on autophagy in NAFLD increases, therapeutic interventions can be developed and tested. Systemic therapy will potentially be hampered if context- and tissue-dependent behaviour of autophagy appears to play an important role in the pathogenesis of the metabolic syndrome. Several clinical trials are already ongoing, almost exclusively in oncological settings (www.clinicaltrials.gov). Investigators should be stimulated to include repetitive evaluation of the metabolic parameters in their study as secondary objectives.

CONCLUSION

Autophagy is an important factor in the lipid metabolism, but its exact role has not yet been fully clarified and appears to be context- and tissue-specific. Increasing knowledge on its exact role in the complex pathophysiology of metabolic disturbances and NAFLD might make autophagy a target for treatment of the metabolic syndrome or NAFLD. We should, however, always keep in mind that altering a key cellular process such as autophagy might lead to a better metabolic state, but that this not automatically equals a better general health.

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